MARGARET R. STEWART, BOBBIE K. HUTCHINS, MARELYNN W. ZIPSER and BETTY M. WATTS
Department of Food and Nutrition, Florida State University, Tallahassee, Florida 32306

Enzymatic Reduction of Metmyoglobin by Ground Beef

SUMMARY

A method is described for estimating the ability of ground meat to reduce metmyoglobin enzymatically. The method involves a complete oxidation of all pigments to metmyoglobin by the addition of ferricyanide, followed by measurement of metmyoglobin reduction by the meat in 1 hr. The work was confined to beef rib eye muscle. Metmyoglobin-reducing activity (MRA) showed great fluctuations in rib eyes from different animals. It was correlated with total pigment. In any one sample of meat, MRA increased with pH from pH 5.1 to 7.1 and with temperature from 3°C to 35°C. It declined only slightly in whole cuts of meat stored for several days in the refrigerator, but much more rapidly in stored ground meat. Chlortetracycline did not interfere with MRA, but 5% salt completely inhibited it. A possible reductive mechanism is discussed briefly.

INTRODUCTION

The maintenance of a desirable color in fresh meat is of interest to both the meat industry and the consumer. Bright-red oxymyoglobin and purplish-red reduced myoglobin are the normal ferrous pigments of fresh meat. The relative proportion of these two depends upon the oxygen tension at the pigment site. Where the pigments are in the ferrous form, exposed meat surfaces quickly assume the bright red of oxymyoglobin. On the other hand, oxidation of the meat pigments to metmyoglobin produces a brown color which is not acceptable to the consumer. Furthermore, there is substantial evidence in the literature that metmyoglobin (but not the ferrous heme pigments) can catalyze lipid oxidation, resulting in flavor changes.

There are numerous reports in the literature on the metmyoglobin discoloration of meats as affected by variations in oxygen tension, storage temperature, packaging materials, bacterial contamination, etc. The metmyoglobin actually formed during such storage studies is a result of two opposing factors, i.e., autoxidation of the ferrous pigments to metmyoglobin, on the one hand, and enzymatic reduction of the ferric metmyoglobin, on the other hand.

Walters and Taylor (1963) demonstrated a slow enzymatic reduction of pure metmyoglobin upon incubation under anaerobic conditions with pork muscle mince at 37°C, pH 6.0. While these appear to be the only published data directly demonstrating metmyoglobin reduction by meat, there is much indirect evidence in meat storage studies. For example, Dean and Ball (1960) showed the disappearance as well as the formation of metmyoglobin on the surfaces of beef cuts stored in various ways and stated that metmyoglobin is converted to oxymyoglobin by "natural processes."

Recent work of Cutaia and Ordal (1964) on ground beef demonstrates even more clearly the initial formation of metmyoglobin and its subsequent disappearance during a two-day storage period under anaerobic conditions. Those authors interpreted their results in terms of simultaneous autoxidation and enzymatic reduction of the meat pigments.

The observation (Stewart *et al.*, 1965) that reduction of metmyoglobin was substantial after ferricyanide treatment of ground beef suggested that this technique might be adapted to a study of the metmyoglobin-reducing activity of meats under various conditions. Thus, enzymatic reduction of preformed metmyoglobin could be separated from the situation of simultaneous oxidation and reduction, which complicates the study of spontaneous pigment changes in meat.

MATERIALS AND METHODS

Preparation of meat. Beef rib eye was trimmed and ground as described previously (Stewart *et al.*, 1965). Fifty-g portions were weighed out and placed in polyethylene freezer bags. If storage was required, samples were placed in a refrigerator at 3°C.

Temperature adjustment. Samples were removed from storage (or freshly prepared) and flattened with a hamburger press to a thickness of ¼ inch while in the bag. The packaged samples were then placed in water baths at various temperatures for 30 min. The standard conditions adopted were 30°C water bath for 30 min.

Ferricyanide addition. Varying amounts of $K_3Fe(CN)_6$ in 2 ml distilled water were added to the 50-g portion, followed by vigorous mixing for 3 min. Spectrophotometric measurements were made as described previously (Stewart *et al.*, 1965). Total elapsed time from the addition of ferricyanide to the start of the first curve was 4½ min. The initial curve was considered to be taken at 0 time, and subsequent curves were taken 30–60 min after this.

Chlortetracycline treatment. A stock solution was prepared of chlortetracycline HCl (American Cyanamide Co.) containing 1 mg per ml .01*N* HCl. One and one-half ml of this solution were added to 50 g meat, followed by thorough mixing. The meat was stored at 3°C and brought to 30° before ferricyanide addition.

pH adjustment. To ascertain the influence of pH on reducing activity, either 1*N* HCl or 5*N* NaOH was added to 50-g portions of meat immediately after grinding. After mixing, the samples were refrigerated for 1 hr and then brought to 30°C before addition of the ferricyanide. The addition of acid or base did not exceed 2 ml/50 g, and the volume of liquid added was kept constant.

RESULTS AND INTERPRETATION

Formation and reduction of metmyoglobin after addition of ferricyanide to meat. Fig. 1 is a photograph of a series of spectra obtained during a period of several hours after the addition of 0.2% potassium ferricyanide to a sample of ground rib eye muscle. The initial curve is that with highest absorption at 635 mμ and lowest at 573 mμ. Subsequent spectra, made at half-hour intervals except for the last two, which are 15 min apart, show a progressive decrease at 635 mμ and increase at 573 mμ. The meat was not removed from its position on the spectrophotometer during this 4¼-hr period. The spectra are recorded against a piece of gray cardboard, whose absorption against the magnesium carbonate reference standard is also shown as the uninflected line in the same figure.

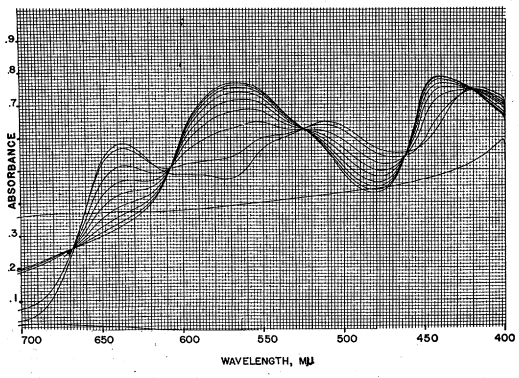

Fig. 1. Reduction of metmyoglobin in rib eye muscle during 4¼ hr.

The addition of ferricyanide resulted in oxidation of the muscle pigments to metmyoglobin. The first curve, recorded 4½ min after addition of the ferricyanide, indicates that a slight amount of oxymyoglobin is present with the metmyoglobin. Consequently, this curve is isobestic with the others only at 525 mμ. A number of isobestic points for reduced and metmyoglobin are clearly shown. The metmyoglobin is progressively reduced by the meat. The K/S ratio at 572 mμ/525 mμ for the final curve is 1.34, compared to an average value of 1.40 obtained with a number of samples of artificially reduced meat (Stewart *et al.*, 1965).

The amount of ferricyanide necessary to get complete oxidation of the pigments varied. With all samples, 0.2% ferricyanide was sufficient, but with meats of relatively low reducing activity 0.1% ferricyanide also oxidized the pigment and the subsequent reduction occurred more rapidly. Fig. 2 shows the reduction of metmyoglobin by two portions of the same sample of ground meat (of moderate reducing activity) to which had been added 0.1% and 0.2% potassium ferricyanide, respectively. With the smaller amount, the pigment was completely oxidized but reduction began immediately. With the larger amount, there was an initial lag in the reduction of metmyoglobin, presumably due to the excess oxidizing agent.

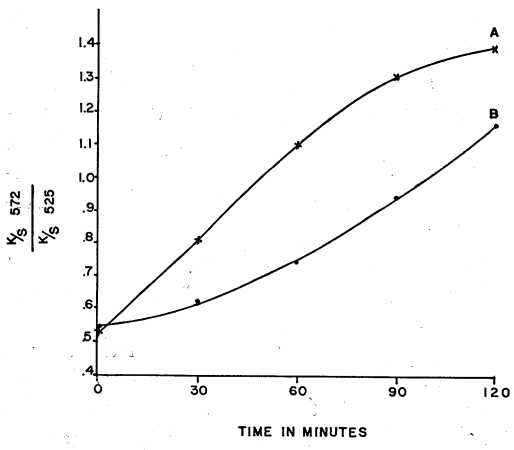

Fig. 2. Influence of ferricyanide concentration on reduction of metmyoglobin. A) 0.1% $K_3Fe(CN)_6$. B) 0.2% $K_3Fe(CN)_6$.

Table 1. Reducing activity of different rib eye samples.

Meat sample	% metmyoglobin [a]	
	0.1% ferricyanide	0.2% ferricyanide
A	100	100
B	48	79
C	43	76
D	28	62
E	12	91
F	2	28

[a] 60 min after $K_3Fe(CN)_6$ addition. Metmyoglobin concentrations obtained from Fig. 2 in preceding paper (Stewart *et al.*, 1965).

When a number of freshly ground samples of rib eye muscle are similarly treated with ferricyanide, the ability of the muscle tissue to reduce the metmyoglobin varies over wide limits. Table 1 summarizes the metmyoglobin remaining 1 hr after addition of ferricyanide to the rib eye muscles from six different animals. The extremes range from no measurable reduction to complete reduction within 1 hr.

A highly significant positive correlation was found between total pigment concentration and reducing activity of tissue. Table 2 lists 8 different rib eye samples in increasing order of hematin content, as estimated from K/S at 525 mμ. Application of the Spearman rank correlation coefficient (Siegel, 1956) gives an rs of + 0.83, significant at the 1% level. This correlation does not necessarily mean a true relation between the pigment and reducing activity. More highly pigmented muscles may also be richer in the enzyme systems responsible for the reaction. The interpretation will have to await further work.

Effect of refrigerator storage. Holding the unground rib eye muscle in the refrigerator for 6 days had only a slight effect on the reducing activity of samples taken from the muscle and ground just before reducing ability was measured. Refrigerator storage of preground samples resulted in much more rapid changes, although the rapidity of the loss varied in different samples of meat. A typical set of data is shown in Table 3. In this and in Table 4 the results are expressed as K/S ratios rather than percent metmyoglobin, because the ratios at zero time with these samples of meat were considerably higher than the average values (Stewart *et al.*, 1965) upon which metmyoglobin percentages were based.

Table 2. Relationship of total pigment concentration to the reducing activity of meat.

Sample	ppm hematin [a]	% metmyoglobin [b]
A	115	83
B	215	100
C	238	79
D	253	62
E	260	30
F	295	17
G	300	7
H	315	38

[a] Hematin concentrations obtained from K/S 525 mμ using Fig. 1 in preceding paper (Stewart *et al.*, 1965).

[b] 60 min after mixing with 0.2% $K_3Fe(CN)_6$. Metmyoglobin concentrations obtained from Fig. 2 in preceding paper (Stewart *et al.*, 1965).

Table 3. Reducing activity of refrigerator-stored ground beef.

Days after grinding of meat [a]	K/S 572 mμ/K/S 525 mμ Minutes after $K_3Fe(CN)_6$ addition		
	0	30	60
0	0.60	1.05	1.30
2	0.62	0.69	0.85
6	0.62	0.63	0.63

[a] pH 5.62; 0.2% $K_3Fe(CN)_6$.

Effect of antibiotics. The addition of 30 ppm chlortetracycline, a broad-spectrum antibiotic, to several samples of ground meats had no effect on the ability of the tissues to reduce metmyoglobin at least up to 3–4 days of storage. The antibiotic could be useful in prolonged storage studies where bacterial effects might obscure the results.

Effect of temperature on reducing activity. Fifty-gram samples of meat were held 30 min at temperatures ranging from 3 to 35°C prior to the addition of 0.2% $K_3Fe(CN)_6$ and during intervals between spectral analyses. The effect of these temperature variations is seen in Table 4.

Little reduction occurred at 15°C or lower, and the rate of reduction increased at least up to 35°C. In other experiments with meat having greater reducing activity than this sample, measurable reduction occurred at 15°C, but, again, the rate was much less than at room temperature. Similar results were reported by Cutaia and Ordal (1964).

As might be expected, cooking meat to 70°C completely destroyed all reducing activity.

Table 4. Effect of temperature on reducing activity.

Sample temperature (°C)	K/S 572 mμ/K/S 525 mμ Minutes after $K_3Fe(CN)_6$ addition		
	0	30	60
3	0.64	0.67	0.68
15	0.64	0.65	0.68
22	0.66	0.85	1.08
35	0.65	1.17	1.26

Effect of pH on reducing activity. Fig. 3 shows the results of an experiment in which the pH of a sample of meat (originally pH 5.8) was adjusted to various values from pH 5.1 to 7.1. The reducing activity becomes progressively higher as the pH increases. The initial K/S ratios vary greatly. The higher initial value for the sample adjusted to pH 5.1 than for those at pH 5.4 or 5.8 is believed to be caused by a difference in texture of the acidified meat. This difference, like that produced by cooking, increases the absorbance of pigment-free meat and so changes the numerical value of the ratios corresponding to the various pigment fractions. On the other hand, the higher initial values of the samples adjusted to pH 6.5 and 7.1 are ascribed to the greatly increased reducing activity of these samples during the time of mixing and placing on the spectrophotometer.

Cutaia and Ordal (1964) have also noted more rapid reduction of metmyoglobin at higher pH. The demonstrated dependence of enzymic reducing activity upon the pH of a given sample of meat does not necessarily mean that a correlation should be expected between the pH and reducing activity of different samples of meat. It is quite possible that a negative rather than a positive correlation could appear if a greater variety of muscles were tested. Berman (1961) found a highly significant inverse correlation between pH and content of the enzyme lactic dehydrogenase.

Effect of added salt. Table 5 demonstrates the inhibitory effect of sodium chloride on the reducing activity of a sample of ground beef. Salt in these concentrations is known to inhibit the activity of many enzymes. Taylor (1961) observed that 5% salt did not interfere with the ability of pork tissue to reduce nitrite to nitric oxide, but the capacity of the tissue to reduce the indigenous pigments to the ferrous nitric oxide myoglobin was appreciably reduced by the salt.

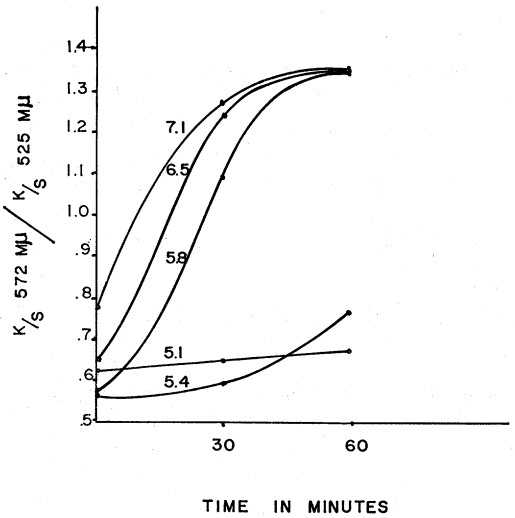

Fig. 3. Reducing activity of rib eye muscle as influenced by pH.

Table 5. Effect of added salt on K/S ratios at 60 minutes.

Sample	K/S 572 mμ/K/S 525 mμ [a]
Control, 0% NaCl	1.15
2% NaCl	0.82
5% NaCl	0.64

[a] 0.1% $K_3Fe(CN)_6$; initial ratio = 0.63.

DISCUSSION

It should be re-emphasized that the reducing activity of meat tissues, investigated here, is only one of several factors involved in the oxidative pigment changes occurring in stored meats. Although increasing the storage temperature, for example, accelerates the enzymatic reduction of metmyoglobin, it also accelerates the myoglobin autoxidation rate. Thus, the net result of a change in temperature (or other environmental factors) on metmyoglobin formation cannot be predicted easily and might not be the same in all meat samples. Great variations are evident in the metmyoglobin-reducing capacity of even a single muscle. Further work on meat of controlled postslaughter treatment from animals of known preslaughter history would be highly desirable.

There is no indication in any of the meat literature of the enzymatic pathways responsible for metmyoglobin reduction. Considering the conditions known to prevail in the postrigor meat, one possible reductive chain might be the following. Hydrogen may be transferred from lactate to diphosphopyridine nucleotide (DPN^+), by the enzyme lactic dehydrogenase (LDH). Meat is well supplied with both lactate (the end product of glycolysis) and LDH (Berman, 1961). The reduced pyridine coenzyme (DPNH) can, in turn, reduce metmyoglobin, but not directly. In work with model systems, Rossi-Fanelli *et al.* (1957) found a requirement for intermediate electron carriers, which may be other enzymes, quinones, methylene blue, etc. It may be postulated that the rate of the over-all reaction is limited either by the con-

centration of DPN+ or by the availability of suitable electron mediators between the reduced coenzyme and metmyoglobin. DPN+ is known to undergo enzymatic hydrolysis in tissue homogenates (Mann and Quastel, 1941; Gutmann, *et al.,* 1947). Obviously it will be necessary to approach the problem at a more fundamental experimental level in order to clarify the enzymatic pathways involved.

REFERENCES

Berman, M. D. 1961. Factors affecting the water retention of beef. V. Variations of the zinc-containing enzymes. *J. Food Sci.* **26**, 422.

Cutaia, A. J., and Z. J. Ordal. 1964. Pigment changes in anaerobically packaged ground beef. *Food Technol.* **18**, 757.

Dean, R. W., and C. O. Ball. 1960. Analysis of the myoglobin fractions on the surfaces of beef cuts. *Food Technol.* **14**, 271.

Gutmann, H. R., B. J. Jandorf, and O. Bodansky. 1947. The role of pyridine nucleotides in the reduction of methemoglobin. *J. Biol. Chem.* **169**, 145.

Mann, P. J. G., and J. H. Quastel. 1941. Nicotinamide, cozymase and tissue metabolism. *Biochem. J.* **35**, 502.

Rossi-Fanelli, A., E. Antonini, and B. Mordovi. 1957. Enzymic reduction of ferrimyoglobin. *Arch. Biochem. Biophys.* **68**, 341.

Siegel, S. 1956. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

Stewart, M. R., M. W. Zipser, and B. M. Watts. 1965. The use of reflectance spectrophotometry for the assay of raw meat pigments. *J. Food Sci.* **30**, 464.

Taylor, A. McM. 1961. Investigation of the biochemical properties of pork muscle as they are related to pigment formation and colour fixation during curing. Second annual report of work done under a grant from the British Food Manufacturing Industries Research Assoc.

Walters, C. L., and A. McM. Taylor. 1963. Biochemical properties of pork muscle in relation to curing. *Food Technol.* **17**, 354.

Ms. rec'd 8/14/64.

This study was supported by Agricultural Research Service, U. S. Department of Agriculture, Grant No. 12-14-100-7655(73) administered by the Eastern Utilization Research and Development Division, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118.